

Screening Assay

The present invention relates to a screening assay, e.g. a method for identifying an agent that interferes with T cell activation and/or -differentiation and/or modulation of other inflammatory effector cells.

T cell receptors (TCRs) play a major role in the recognition of antigens and the subsequent immune response. Cytotoxic CD8⁺ T lymphocytes recognize antigens as peptide fragments in association with MHC class I molecules on the cell surface, whereas CD4⁺ T lymphocytes recognize antigens as peptide fragments in association with MHC class II molecules on the cell surface. The specific recognition induces a series of events including clonal expansion and the modulation (activation) of surface adhesion molecules such as, e.g. CD69, CD25, CD44, CD62, CD71 and CD11a in case of CD8⁺ T cells. Ultimately, in CD8⁺ T cells such changes may lead to cell differentiation into cytotoxic effector cells (=Th 1 cells), whereas in CD4⁺ T cells differentiation into T helper cells (=Th 2 cells) occurs, both capable of promoting T cell dependent immunity.

Activation of T cells may be assayed by determining either the amount of certain cell surface molecules or the presence of such molecules, which are up-regulated upon activation, such as e.g. CD25 and CD69 on peripheral blood lymphocytes and spleen cells in case of CD8⁺ T cells, or by measuring the amount of T-cell mediators (lymphokines), which are produced (released) upon activation. These mediators are able to activate or prime other cells, e.g. monocyte/macrophages, for enhanced release of pro-inflammatory and anti-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, IL-10 or TNF- α , respectively.

Cytokines may induce a complex spectrum of biological responses that are generally thought to regulate host defense. A subset of cytokines, including e.g. interleukin-1 (IL-1 α and IL-1 β) and tumor necrosis factor (TNF- α), mediate biological actions consistent with a role as inflammatory mediators. The temporary or sustained release of these pro-inflammatory cytokines plays a critical role in the pathogenesis of acute and chronic inflammation in several diseases e.g. autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, systemic Lupus, psoriasis, multiple sclerosis and others.

But determining the amount of such T-cell and/or inflammatory effector cell derived mediators in vivo is difficult.

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E.g. in normal mice the frequency of cells responding to a given antigen is usually below 1 in 100,000 lymphocytes and cytokine levels and/or levels of other mediators in serum are below the detection limit of assays. For testing potential drugs for their T-cell and/or other inflammatory effector cell activity in vivo, the use of an animal model in which levels of the mediators of interest can be determined in serum would be advantageous.

We have now surprisingly found a convenient possibility for the determination of T-cell and/or inflammatory effector cell activity, e.g. T-cell and/or inflammatory effector cell derived mediators, directly in vivo in serum.

In one aspect the present invention provides an kit (assay) for the determination of T-cell and/or inflammatory effector cell derived mediators directly in vivo in serum, comprising a mouse wherein the majority of T cells express a transgenic MHC class I restricted or MHC class II restricted T cell receptor.

T-cell and inflammatory effector cell derived mediators, such as mediators induced downstream of the lymphokine production, include e.g. cytokines, chemokines and/or other mediators, which are produced (released) upon T-cell and inflammatory effector cell activation, preferably cytokines and chemokines, such as selected from the group consisting of IL1- α , IL-1 β , IL-2, IL-4, IL-6, IL-10, TNF- α and IFN- γ .

Mice wherein the majority of T cells express a transgenic MHC class I restricted or MHC class II restricted T cell receptor, such as transgenic mice expressing an ovalbumin (OVA-peptide)-specific TCR, e.g. include DO11.10 mice or OT-1 mice. OT-1 mice may be obtained e.g. as described in Hogquist et al., 1994, Cell 76(1)17-27, DO11.10 mice may be obtained e.g. as described in Murphy K.M. et al. 1990, Science 250:1720-1722.

In an OT-1 mouse with the specificity for a MHC class I restricted peptide, the MHC class I restricted peptide is preferably a (restricted) ovalbumin(OVA)-peptide, e.g. OVA₂₅₇₋₂₆₄. In a DO11.10 mouse with a specificity for a MHC class II restricted peptide, the MHC class II restricted peptide is preferably a (restricted) ovalbumin peptide, e.g. OVA₃₂₃₋₃₃₉.

In more detail, we have found that transgenic mice expressing an ovalbumin (OVA)-specific transgenic TCR, e.g. DO11.10 mice or OT-1 mice, and thus containing a high percentage of OVA-specific T cells e.g. in their lymphoid tissue, after stimulation with an appropriate stimulation agent, e.g. by administration of an antigen to the ovalbumin (OVA-peptide)-

specific TCR, such as the OVA₃₂₃₋₃₃₉ peptide in DO 11.10 mice or the OVA₂₅₇₋₂₆₄ peptide in OT-1 mice, release T-cell and/or inflammatory effector cell derived mediator amounts which may be easily detectable by conventional assays.

We also have found that TCR transgenic mice, e.g. DO11.10 or OT-1 mice, may produce substantial amounts of IFN- γ upon stimulation by administration of (restricted) OVA-peptides with the respective TCR specificity. We further have found that in peptide stimulated DO11.10 mice endogenous production of IFN- γ before challenge with a triggering agent, such as an endotoxin, may even potentiate the production of pro-inflammatory cytokines such as e.g. IL-1 α , IL-1 β and TNF- α .

In another aspect the present invention provides a kit (assay) of the present invention comprising as main components

- a. a mouse wherein the majority of T cells express a transgenic MHC class I restricted or MHC class II restricted T cell receptor,
- b. a MHC restricted TCR specific OVA-peptide,
- c. optionally a triggering agent,
- d. optionally controls, standards and/or calibration means,
- e. optionally detection means for a T-cell and/or inflammatory effector cell derived mediator, and
- f. instructions for using the components of said kit.

A kit (assay) of the present invention may further comprise a substantial component, e.g. including an appropriate environment of a sample to be tested.

Appropriate T-cell and/or inflammatory effector cell derived mediator-detection means

include detection means according to a method as conventional, such as mediator detection with immunoassays such as e.g. all sorts of ELISAs (=enzyme linked immunosorbent assay), e.g. with an antibody which bears a label for detection or which bears no label. Such a label may be as conventional, e.g. an enzyme label, such as horse radish peroxidase (HRP) or peroxidase (POD); or a fluorescence label, e.g. a fluorescent dye. Such label may be detected as appropriate, e.g. according to a method as conventional, e.g. enzyme detection methods by carrying out an enzymatic reaction, or by fluorescence measurement. An antibody which bears no label may be detected as appropriate, e.g. indirectly, such as by use of a tandem system of a first antibody and a second antibody specifically recognizing the first antibody, which second antibody bears a label, e.g. an enzyme or fluorescence label, the

label of which may be detected according to methods as conventional, e.g. as indicated herein.

A MHC restricted TCR specific OVA-peptide is preferably administered parenterally, e.g. intraperitoneal, intravenous or subcutaneous.

5 A triggering agent is understood to be an agent which stimulates the enhanced production of non-T-cell derived mediators, e.g. other inflammatory effector cell derived mediators, in a TCR transgenic mouse when stimulated before with the T specific peptide. An appropriate triggering agent includes a triggering agent as conventional, such as an endotoxin or a lipopolysaccharide of gram negative bacteria (LPS).

10 If a triggering agent is administered, the serum levels of pro-inflammatory and anti-inflammatory cytokines are determined, preferably the levels of IL-1 α and/or IL-1 β and/or IL-6 and/or TNF- α and/or IL-10.

In an OT-1 mouse with the specificity for a MHC class I restricted TCR specific peptide, the peptide is preferably OVA₂₅₇₋₂₆₄. The T cell mediator determined in an OT-1 mouse is
15 preferably a CD8⁺ T cell derived cytokine, e.g. IL-2 and/or IFN- γ .

In a DO11.10 mouse with a specificity for a MHC class II restricted TCR specific peptide, the peptide is preferably OVA₃₂₃₋₃₃₉. The T cell mediator determined in a DO11.10 mouse is preferably a CD4⁺ T cell derived cytokine, e.g. one selected from the group consisting of IL-2, IL-4, IL-5, IL-13 and IFN- γ .

20 In another aspect the present invention provides a process for the determination of a T-cell and/or inflammatory effector cell derived mediator in serum of an OVA-peptide stimulated mouse wherein the majority of T cells express a transgenic MHC class I or MHC class II restricted T cell receptor, which process comprises the steps of

- 25 a. administering to a mouse wherein the majority of T cells express a transgenic MHC class I restricted or MHC class II restricted T cell receptor a MHC restricted TCR specific OVA-peptide and optionally a triggering agent, and
b. determining the level of a T-cell and/or inflammatory effector cell derived mediator produced.

30 If a DO11.10 mouse and a MHC restricted TCR specific OVA-peptide are used in a process of the present invention, preferably the mediator determined is a cytokine selected from the group consisting of IL-2, IL-4, IL-5, IL-13 and IFN- γ ; and if additionally a triggering agent is

administered, preferably the mediator determined is a cytokine selected from the group consisting of IL-1 α , IL-1 β , IL-6, IL-10 and TNF- α .

5 A kit or a process of the present invention may be useful in the provision of an agent that interferes with T cell activation and/or -differentiation and/or modulation of other inflammatory effector cells in vivo, which agent may be useful as a pharmaceutically active compound.

In another aspect the present invention provides a method for identifying, e.g. and using as a pharmaceutically active compound, an agent that interferes with T cell activation and/or -

10 differentiation and/or modulation of other inflammatory effector cells comprising the steps of

a. administering to a mouse wherein the majority of T cells express a transgenic MHC class I or MHC class II restricted T cell receptor a MHC restricted TCR specific OVA-peptide and optionally a triggering agent,

15 b. administering to a transgenic mouse of step a) a candidate compound before, after or simultaneously with the peptide and optionally a triggering agent of step a),

c. determining the level of a T-cell and/or inflammatory effector cell derived mediator in serum of

c1. a mouse treated according to step a), and

c2. a mouse treated according to step b) and step a),

20 d. determining whether there is a difference in the level of said mediator produced in said serum, and

e. choosing an agent as determined in step d).

25 A candidate compound includes compound (libraries), from which the effect on T cell activation and/or -differentiation and/or modulation of other inflammatory effector cells is unknown. Compound (libraries) include for example oligopeptides, polypeptides, proteins, antibodies, (peptide-)mimetics, small molecules, e.g. low molecular weight compounds (LMW's). An agent is a candidate compound from which an effect on T cell activation and/or -differentiation and/or modulation of other inflammatory effector cells has been found in a

30 screening assay or in a method for identifying an agent of the present invention. An agent may decrease or enhance the production of mediators from T cells or other inflammatory effector cells and includes agonists and antagonists in the production of such mediators. An agent includes compound(s)(libraries) from which its influence on T cells, e.g. CD8⁺ T cells and/or CD4⁺ T cells, and/or other inflammatory effector cells, e.g. monocytes/macrophages,

via its influence on the release of appropriate mediators in vivo, can be determined.

Compound (libraries) include for example oligopeptides, polypeptides, proteins, antibodies, (peptide-)mimetics, small molecules, e.g. low molecular weight compounds (LMW's).

In case a candidate compound is administered, the candidate compound is preferably
5 administered orally or parenterally.

In another aspect the present invention provides

- a mouse wherein the majority of T cells express a transgenic MHC class I or MHC class II
10 restricted T cell receptor for use in a method for the identification of an agent that interferes
with T cell activation and/or -differentiation and/or modulation of other inflammatory effector
cells;
- the use of a mouse wherein the majority of T cells express a transgenic MHC class I or
MHC class II restricted T cell receptor in a method for the identification of an agent that
interferes with T cell activation and/or -differentiation and/or modulation of other
15 inflammatory effector cells,
e.g. comprising steps a. to e. as defined above.

In another aspect the present invention provides

- the use of a mouse wherein the majority of T cells express a transgenic MHC class I or
20 MHC class II restricted T cell receptor in a method for the determination of a T-cell and/or
inflammatory effector cell derived mediator in the serum of said mouse;
e.g. comprising the steps of a process for the determination of a T-cell and/or inflammatory
effector cell derived mediator of the present invention.

- 25 In another aspect the present invention provides a kit of the present invention for use in a
method for identifying an agent that interferes with T cell activation and/or -differentiation
and/or modulation of other inflammatory effector cells.

Said kit may further comprise a substantial component, e.g. including an appropriate
environment of a sample to be tested and, e.g. appropriate means to determine the effect of
30 a candidate compound in a sample to be tested.

In another aspect the present invention provides an agent identified by a method comprising
the steps of

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- a. administering to a mouse wherein the majority of T cells express a transgenic MHC class I or MHC class II restricted T cell receptor a MHC restricted TCR specific OVA-peptide and optionally a triggering agent,
- b. administering to a transgenic mouse of step a) a candidate compound before, after or simultaneously with the peptide and optionally a triggering agent of step a),
- c. determining the level of a T-cell and/or inflammatory effector cell derived mediator in serum of
 - c1. a mouse treated according to step a) and
 - c2. a mouse treated according to step b) and step a),
- d. determining whether there is a difference in the level of said mediator produced in said serum, and
- e. choosing an agent as determined in step d).

An agent of the present invention may exhibit pharmacological activity and is therefore useful as a pharmaceutical, e.g. in the treatment of diseases which are mediated by T-cell and/or inflammatory effector cell derived mediators. Inhibitory agents might be useful as a pharmaceutical for e.g. diseases based on an unwanted or aberrant immune response, such as allergic diseases, transplantation, autoimmune related and inflammatory diseases. Allergic disease include e.g. allergic asthma, contact allergy, drug allergy, food allergy, atopic dermatitis or seasonal allergies such as allergic rhinitis. Transplantation includes transplantation of solid organs, skin, cornea transplantation or bone marrow transplantation. Autoimmune related and inflammatory diseases include e.g. type I diabetes, multiple sclerosis, rheumatoid arthritis, psoriasis, inflammatory bowel disease, atherosclerosis or systemic Lupus erythematosus.

Stimulatory agents might be useful in all situations where the immune response should be strengthened, e.g. infections, immunodeficiencies or tumors.

In another aspect the present invention provides

- an agent of the present invention for use as a pharmaceutical, e.g. for the treatment of a disease which is based on an unwanted or aberrant immune response selected from the group consisting of allergic disease, transplantation, autoimmune related disease, inflammatory disease and modulation/stimulation of a tumor specific or pathogen specific response, and
- a pharmaceutical composition comprising at least one agent that interferes with T cell

activation and/or –differentiation and/or modulation of other inflammatory effector cell identified by a method according to the present invention beside pharmaceutically acceptable excipient(s).

- 5 Pharmaceutically acceptable excipient(s) include e.g. appropriate carrier and/or diluent, e.g. including fillers, binders, disintegrators, flow conditioners, lubricants, sugars and sweeteners, fragrances, preservatives, stabilizers, wetting agents and/or emulsifiers, solubilizers, salts for regulating osmotic pressure and/or buffers.
- 10 In another aspect the present invention provides a method for the treatment of a disease which is based on an unwanted or aberrant immune response selected from the group consisting of allergic disease, transplantation, autoimmune related disease, inflammatory disease and modulation/stimulation of a tumor specific or pathogen specific response, comprising administering an agent identified by a method of the present invention or a
- 15 pharmaceutical composition of the present invention to a subject in need of such a treatment.

In another aspect the present invention provides a method for the treatment of a disease which is based on an unwanted or aberrant immune response selected from the group consisting of allergic disease, transplantation, autoimmune related disease, inflammatory

20 disease and modulation/stimulation of a tumor specific or pathogen specific response, wherein the

- allergic disease is allergic asthma, contact allergy, drug allergy, food allergy, atopic dermatitis or seasonal allergy,
- transplantation is transplantation of solid organs, skin, cornea or bone marrow,
- 25 - the autoimmune related and inflammatory disease is type I diabetes, multiple sclerosis, rheumatoid arthritis, psoriasis, inflammatory bowel disease, atherosclerosis or systemic Lupus erythematosus,
- the tumor specific or pathogen specific response is infection, immunodeficiencies or tumor treatment.

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An agent of the present invention for treatment includes one or more, preferably one, agent of the present invention, e.g. a combination of two or more agents of the present invention. Treatment includes treatment and prophylaxis.

In another aspect the present invention provides the use of an agent of the present invention for the manufacture of a medicament, e.g. a pharmaceutical composition, for the treatment of diseases which are mediated by T-cell and/or inflammatory effector cell derived mediators, e.g. diseases based on an unwanted or aberrant immune response, such as allergic diseases, transplantation, autoimmune related and inflammatory diseases or in situations where the immune response should be strengthened, e.g. infections, immunodeficiencies, tumors and the use as vaccines (adjuvants).

For such treatment (use), the appropriate dosage will, of course, vary depending upon, for example, the chemical nature and the pharmacokinetic data of an agent of the present invention employed, the individual host, the mode of administration and the nature and severity of the conditions being treated. However, in general, for satisfactory results in larger mammals, for example humans, an indicated daily dosage is in the range from about 0.01 g to about 2.0 g (e.g. about 0.125 mg/kg to about 25 mg/kg), such as 0.05 to 2.0 g, e.g. 0.1 to 0.5 mg, of an agent of the present invention; conveniently administered, for example, in divided doses up to four times a day.

An agent of the present invention may be administered by any conventional route, for example enterally, e.g. including nasal, buccal, rectal, oral administration; parenterally, e.g. including intravenous, intramuscular, subcutaneous administration; or topically; e.g. including epicutaneous, intranasal, intratracheal administration; e.g. in form of coated or uncoated tablets, capsules, injectable solutions or suspensions, e.g. in the form of ampoules, vials, in the form of creams, gels, pastes, inhaler powder, foams, tinctures, lip sticks, drops, sprays, or in the form of suppositories.

An agent of the present invention may be administered in the form of a pharmaceutically acceptable salt, e.g. an acid addition salt or metal salt; or in free form; optionally in the form of a solvate. An agent of the present invention in the form of a salt may exhibit the same order of activity as an agent of the present invention in free form; optionally in the form of a solvate.

An agent of the present invention may be used for pharmaceutical treatment according to the present invention alone, or in combination with one or more other pharmaceutically active agents.

Combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents

in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

- 5 In another aspect the present invention provides a pharmaceutical composition according to the present invention, further comprising another pharmaceutically active agent.

Such compositions may be manufactured according, e.g. analogously to a method as conventional, e.g. by mixing, granulating, coating, dissolving or lyophilizing processes. Unit
10 dosage forms may contain, for example, from about 0.5 mg to about 2000 mg, such as 1 mg to about 500 mg, e.g. 0.00625 mg/kg to about 12.5 mg/kg.

In another aspect the present invention provides a method for identifying, e.g. and using as a pharmaceutical, an agent that interferes with T-cell activation and/or differentiation, esp. with
15 CD8⁺ T-cells, comprising the steps of

- a) providing a mouse wherein the majority of T-cells express a transgenic MHC class I restricted T-cell receptor, e.g. an OT-1 mouse,
- b) administering to a transgenic T-cell receptor carrying mouse according to step a) the
20 respective T-cell receptor specific peptide, e.g. OVA₂₅₇₋₂₆₄ peptide in an OT-1 mouse, in a defined amount,
- c) administering to a transgenic mouse of step b) a candidate compound which might interfere with T-cell activation/differentiation, esp. with CD8⁺ T-cells, before, after or simultaneous with the peptide of step b), in a defined amount,
- d) determining the level of a T-cell derived cytokine, e.g. a CD8⁺ T-cell derived cytokine,
25 preferably IL-2 and IFN- γ , in serum of candidate compound treated mouse according to steps a) – c) versus candidate compound untreated mouse according to steps a) and b) and determining whether there is a difference in the cytokine level, and
- e) choosing an agent from said candidate compound as determined in step d), e.g. for use as a pharmaceutical.

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In another aspect the present invention provides a method for identifying, e.g. and using as a pharmaceutical, an agent that interferes with T-cell activation and/or differentiation, esp. with CD4⁺ T-cells, comprising the steps of

- a) providing a mouse wherein the majority of T-cells express a transgenic MHC class II

- restricted specific T-cell receptor, e.g. a DO11.10 mouse,
- b) administering to a transgenic T-cell receptor carrying mouse according to step a) the respective T-cell receptor specific peptide, e.g. OVA₃₂₃₋₃₃₉ peptide in a DO11.10 mouse, in a defined amount,
- 5 c) administering to a transgenic mouse according to step b) a candidate compound which might interfere with T-cell activation and/or differentiation, esp. with CD4⁺ T-cells, before, after or simultaneous with the peptide of step b), in a defined amount,
- d) determining the level of a T-cell derived cytokine, e.g. a CD4⁺ T-cell derived cytokine, preferably IL-2, IL-4 and/or INF- γ , in serum of candidate compound treated mouse
- 10 according to steps a) – c) versus candidate compound untreated mouse according to steps a) and b) and determining whether there is a difference in the cytokine level, and
- e) choosing an agent from said candidate compound as determined in step d), e.g. for use as a pharmaceutical.
- 15 In another aspect the present invention provides a method for identifying, e.g. and using as a pharmaceutical, an agent that interferes with T cell derived cytokine mediated cell activation, comprising the steps of
- a) providing a mouse wherein the majority of T-cells express a transgenic MHC class II or MHC class I restricted T-cell receptor, e.g. a DO11.10 or an OT-1 mouse,
- 20 b) administering to a transgenic mouse according to step a) a T-cell receptor specific peptide as a priming agent, e.g. OVA₃₂₃₋₃₃₉ peptide in a DO11.10 mouse or OVA₂₅₇₋₂₆₄ peptide in an OT-1 mouse, in a defined amount,
- c) administering to a transgenic mouse according to steps a) and b) a candidate compound which might interfere with T cell derived cytokine mediated cell activation before, after or
- 25 simultaneous with the priming agent of step b) in a defined amount,
- d) administering to a transgenic mouse of steps a) to c) as a triggering agent an endotoxin/lipopolysaccharide from gram negative bacteria in a defined amount,
- e) determining the level of an inflammatory cytokine and/or chemokine and/or another mediator, preferably IL-1 α , IL-1 β , IL-6, TNF- α and/or IL-10, in serum of candidate
- 30 compound treated mouse according to steps a) – d) versus candidate compound untreated mouse according to steps a), b) and d) and determining whether there is a difference in a cytokine and/or chemokine and/or another mediator level,
- f) choosing an agent from said candidate compound as determined in step e), e.g. for use as a pharmaceutical.

DESCRIPTION OF THE FIGURES

Figure 1: Groups of sex-matched OT-1 mice are given 100 µg/mouse of OVA₂₅₇₋₂₆₄ in 0.2 ml PBS i.p.. At indicated time points, peripheral blood and splenocytes are harvested and stained for the expression of activation markers and on CD8⁺ T cells. Data is shown as values of individual mice.

Figure 2: Groups of sex-matched OT-1 mice are given indicated amounts of OVA₂₅₇₋₂₆₄ in 0.2 ml PBS i.p. 4 hours later, peripheral blood is collected and serum is analyzed for IFN-γ and IL-2. Data is expressed as values of individual mice.

Figure 3: Groups of sex-matched OT-1 mice are given CsA p.o. in Neoral placebo at the indicated dose. 2 hours after compound administration, animals are immunized with 30 µg/mouse of OVA₂₅₇₋₂₆₄ in 0.2 ml PBS i.p. The negative control group (PBS) receive 0.2 ml PBS i.p. only. After 4 hours blood stained for the expression of activation markers CD25 (left) and CD69 (right) on CD8⁺ T cells. Data is expressed as individual values per mouse with a horizontal bar giving the mean value per group. Stars indicate significant differences (p<0.05) to the vehicle-treated, peptide-primed control group (Peptide). § denotes a mouse that was poorly primed.

Figure 4: shows the corresponding effect on the serum levels of IFN-γ and IL-2 after CsA treatment. Interestingly, a low dose of CsA (0.1 mg/kg b.w.) leads to an increase in both IFN-γ as well as IL-2 levels, whereas an increased amount of CsA (10 mg/kg b.w.) leads to the complete abrogation of the cytokines in the serum.

In the following examples temperatures are given in degree Celcius (°C) and are uncorrected.

The following ABBREVIATIONS are used:

b.w.	body weight
FACS	fluorescence-activated cell sorting
IFN-γ	interferon-γ
IL	interleukin
i.p.	intraperitoneal

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	mAb	monoclonal antibody
	CsA	cyclosporin A
	LPS	lipopolysaccharide
	OVA	chicken ovalbumin
5	OVA ₂₅₇₋₂₆₄	peptide derived from chicken ovalbumin containing amino acids from position 257 to 264
	OVA ₃₂₃₋₃₃₉	peptide derived from chicken ovalbumin containing amino acids from position 323 to 339
10	PE	phycoerythrin
	FITC	fluorescein isothiocyanate
	TCR	T cell receptor
	TMB	tetramethylbenzidine
	TNF- α	tumor necrosis factor- α
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EXAMPLES**EXAMPLE 1: OT-1 mice****A) EXPERIMENTAL SETUP****a) Laboratory animals**

- 5 OT-1 mice are obtained as described in Hogquist et al., 1994, Cell 76(1)17-27. The animals are maintained in Microvent positive air supply cages ($22\pm 1^{\circ}\text{C}$, $55\pm 5\%$ relative humidity, 50-70 changes of fresh air/hour and 12 hours day and night cycle) with sawdust bedding. Standard laboratory mouse chow (SNIFF, Soest, Germany) and drinking water is given *ad libitum*. Groups of two to four, 8-10 weeks old, male or female OT-1 mice weighing between
- 10 20-25 g are used for the experiments described. The animals are grouped (4 per group) in type II Macrolon cages one day before the experiment.

b) T cell proliferation assay

- Splenocytes (4×10^5 cells/well) of OT-1 mice are stimulated in 200 μl final volume in 96-well flat bottomed plates with titrated amounts of OVA or OVA₂₅₇₋₂₆₄ peptide at 37° . As medium
- 15 RPMI 1640 supplemented with 10% FCS, 2.0 g/l NaHCO_3 , 2 mM L-glutamin, 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 5×10^{-5} M β -mercaptoethanol (Sigma, Vienna, Austria) is used. After 48 hours, cells are pulsed with 0.5 μCi ^3H -thymidine for 8 hours and the incorporated radioactivity is measured on a liquid scintillation β -counter (Trilux, 1450 Microbeta, Wallac, Turku, Finland).

20 c) Cytokine production assay

- Cells are stimulated as described above and supernatant is harvested after 48 hours. The quantity of cytokines released by T cells into the culture medium after antigen stimulation is assayed by conventional sandwich ELISA. Microtiter plates (Nunc-ImmunoplateTM) are coated with the cytokine-specific capture antibodies JES6-1A12 (anti-IL-2), 11B11 (anti-IL-4),
- 25 TRFK5 (anti-IL-5), JES5-2A5 (anti-IL-10) or R4-6A2 (anti-IFN- γ) at 4° overnight. Supernatants are incubated on the coated plates for 2 hours. Specifically bound cytokines are quantified using biotinylated secondary antibodies JES6-5H4 (anti-IL-2), BVD6-24G2 (anti-IL-4), TRFK4 (anti-IL-5), SXC-1 (anti-IL-10) and XMG1.2 (anti-IFN- γ) followed by extravidin peroxidase and TMB substrate (both from Sigma). Absorption is measured at 450
- 30 and 690 nm on a Spectramax 340PC reader (Molecular Devices, Sunnyvale, CA). All cytokines are quantified with standard curves obtained with known amounts of recombinant mouse cytokines using SoftMax 3 software. All mAbs and recombinant mouse cytokine standards are purchased from BD PharMingen, Heidelberg, Germany.

d) Treatment and cell preparation

CsA is used as Neoral® (commercial drinking solution 100 mg/ml Novartis Pharma GmbH, Vienna). Doses of 3, 0.3 and 0.1 mg/kg b.w. are administered orally by gavage 2 hours before antigen challenge. The compound is diluted with solvent (Neoral Placebo) and given in a volume of 0.2 ml. Animals in the control group receive the solvent only. Ovalbumin-derived peptide (OVA₂₅₇₋₂₆₄, SIINFEKL, >80% purity, piChemie R&D, Graz, Austria) or whole OVA (Sigma) are used as antigens. Both are diluted in PBS and administered i.p. in a final volume of 0.2 ml. At various time points after OVA₂₅₇₋₂₆₄ peptide injection about 200 µl blood is withdrawn from the retro-orbital plexus under ether anesthesia. In order to avoid coagulation, blood is collected in EDTA-containing buffer. Mice are killed by cervical dislocation and the spleen is excised. Single cell suspension are prepared in PBS by gently pressing the spleen through a 100 µm cell strainer. Finally, blood and spleen cells are washed with FACS buffer (PBS, 1g/l EDTA, 1g/l NaN₃, 10% FCS; pH 7.1) and taken up in the same buffer for FACS analysis.

e) FACS analysis

Single cell suspensions of spleen or blood containing approximately 1×10^6 cells in 100 µl of FACS buffer are used for staining. Non-specific binding is blocked by addition of 2 µl of unlabeled Fcγ III/II receptor-blocking Ab (anti-CD16/CD32, clone CT-17.1, 17.2). For phenotypic analysis, samples are stained with PE-labeled anti-CD8 mAb (clone CT-CD8a) in conjunction with FITC-labeled anti-CD4 mAb (clone CT-CD4), PE-labeled anti-CD8 mAb in conjunction with FITC-labeled anti-TCR Vβ5.1,5.2 antibody (clone MR9-4) or FITC-labeled anti-CD8 mAb in conjunction with PE-labeled anti-TCR Vα2 antibody (clone B20.1). For detection of activation markers cells are stained with FITC-labeled anti-CD8 mAb (clone CT-CD8a) in conjunction with either PE-labeled anti-CD25 mAb (clone PC61 5.3) or PE-labeled anti-CD25 mAb (clone H1.2F3). All antibodies except for anti-TCR Vβ5 and Vα2 are obtained from Caltag, Burlingame, CA. Those are purchased from BD PharMingen. Blood cells are treated after antibody staining with NH₄Cl buffer (PharM Lyse, BD PharMingen). Cells are washed in FACS buffer and data is acquired on a FACS Calibur (Becton Dickinson) flow cytometer using Cellquest Plus software. Lymphocytes are gated in the FSC/SSC dot blot according to size and granularity and further analyzed for expression of CD8, TCR Vβ5 expression and activation markers. Data are presented as original dot blots or are calculated from dot blots as percentage of cells positively stained for TCR or activation markers within the CD8⁺ population.

Data calculated from FACS analysis or serum cytokine determination are given as individual values for each animal. Data are analysed for normal distribution and then the ANOVA test

for multiple comparison is applied using SigmaStat software. Statistical significance is taken as $p < 0.05$.

B) RESULTS

a) Kinetics of peptide-induced T cell activation in vivo

- 5 Upon i.p. treatment of mice with 100 μg of the immunogenic peptide OVA₂₅₇₋₂₆₄ the frequency of CD8⁺ expressing cells among the lymphocyte population in the blood drops from around 30% to less than 2.5% within 3 hours. CD8⁺ T cell numbers remain at this low level for 24 hours and then recover to nearly pre-experiment levels after 48 hours. In the spleen no significant decrease of CD8⁺ T cells is observed.
- 10 Administration of 100 μg of OVA₂₅₇₋₂₆₄ induces the up-regulation of CD25 and CD69 on both splenic CD8⁺ T cells and the low number of remaining blood CD8⁺ T cells. CD25 is detectable after 3 hours, maximal (ca. 40-fold increase) after 7 hours and reaches background levels after 48 hours. CD69 induction is more rapid with maximal levels (ca. 155-fold increase) reached already after 3 hours and is sustained for more than 24 hours.
- 15 Antigen-mediated activation by peptide injection does not lead to a significant down regulation of the transgenic TCR (see Figure 1).
- Peptide-induced activation in OT-1 mice is accompanied by a transient burst of the inflammatory cytokines IFN- γ and IL-2 in the serum. Both cytokines show an early peak at 4 hours, weaning off by 8 hours and reach background levels after 24 hours.
- 20 Although high amounts of OVA protein induce proliferation and cytokine release of OT-1 splenocytes in vitro, i.p. administration of 1 mg of OVA results only in a late (7-24 hours) and low increase (max. 2.5-fold) of CD69 expression in the spleen but not the blood. The frequency of CD8⁺ T cells in both spleen and blood declines marginally over 48 hours.

b) Dependence of activation on peptide amounts

- 25 The influence of antigen amount on CD8⁺ T cell activation is determined in order to estimate the threshold concentration for activation in vivo and to establish an optimal amount for a routine model (see Figure 2). A minimal amount of 30 μg of OVA₂₅₇₋₂₆₄ per animal is required to induce significant serum quantities of both IFN- γ and IL-2.
- The extent of T cell activation also correlates with the administered peptide amount with a
- 30 maximal response at 30 μg of OVA₂₅₇₋₂₆₄ per animal.

c) Effect of CsA on peptide-induced T cell activation

In order to validate the model, various amounts of immunosuppressant CsA are used to interfere with T cell activation. The spleen is chosen as the target organ for analysis of activation and the peptide is given in an amount of 30 μg /mouse. Because maximal

activation is already seen after 4 hours post peptide administration, this time point is selected for analysis. As shown in **Figure 3**, CsA significantly inhibits peptide-induced CD25 expression at a dose of 1 mg/kg b.w. ($30.1\% \pm 5.2\%$ inhibition) and 10 mg/kg b.w. ($49.5\% \pm 10\%$ inhibition). After 4 hours blood is taken and serum analyzed for IFN- γ and IL-2 in a sandwich ELISA (see **Figure 4**).

EXAMPLE 2: DO11.10 mice

A) EXPERIMENTAL SETUP

a) Laboratory animals

Groups of six, 8-10 weeks old, male or female DO11.10 mice weighing between 20-22 g are obtained as described in Murphy K.M. et al. 1990, Science 250:1720-1722. The animals are maintained in Microvent positive air supply cages ($22 \pm 1^\circ \text{C}$, $55 \pm 5\%$ relative humidity, 50-70 changes of fresh air/hour and 12 hours day and night cycle) with sawdust bedding. Standard laboratory mouse chow (SNIFF, Soest, Germany) and drinking water is given *ad libidum*.

The animals are used for the study after an adaptation period of 10-14 days and grouped (6 per group) in type II Macrolon cages one day before the experiment.

b) Administration of OVA or OVA₃₂₃₋₃₃₉ peptide

Groups of six DO11.10 mice are i.p. injected with 10 μg of ovalbumin (OVA, Sigma, St. Louis, USA) absorbed to 2 mg of $\text{Al}(\text{OH})_3$ (Alu Gel S; 2% $\text{Al}(\text{OH})_3$, Serva, Heidelberg, FRG) and diluted with saline to a volume of 0.2 ml or various amounts (1, 3, 10, 30, or 90 μg per mouse) of alum-precipitated OVA₃₂₃₋₃₃₉ peptide (98% purity, piChem R&D, Graz Austria) in 0.2 ml on days 0 and 14. Before and at different time points on days 0 and 14 after the OVA or OVA₃₂₃₋₃₃₉ peptide administration about 200 μl of blood are collected by puncture of the retro-orbital plexus under ether anesthesia.

The sera are collected and stored frozen at -20°C until analyzed.

c) Treatment with CsA

CsA (Neoral 100 mg/ml Novartis Pharma GmbH, Vienna) is administered orally by gavage. The Neoral solution is diluted further with tap water. Doses of 20, 40, and 80 mg/kg b.w. are administered in a volume of 0.2 ml. Animals in the control group receive the solvent alone.

d) Quantification of cytokines in mouse sera

Murine IL-2, IFN- γ and IL-4 are analyzed by immunoassays (Quantikine R&D Systems, Minneapolis, MN, USA). Results are expressed as pg per ml serum expressed as mean \pm SEM pg of the cytokine indicated per ml serum. Test and control groups are evaluated by Dunn test by Kruskal-Wallis One Way Anova using the SigmaStat® program.

B) RESULTS**a) Sensitization with OVA₃₂₃₋₃₃₉ peptide versus OVA**

Serum INF- γ levels in DO11.10 mice after sensitization with OVA protein (=OVA) or OVA₃₂₃₋₃₃₉ peptide are shown in **TABLE 1**. On days 0 and 14 groups of 6 DO11.10 mice are injected i.p. either with alum/alum (a/a), 10 μ g OVA/10 μ g OVA (o/o), 10 μ g OVA/10 μ g OVA₃₂₃₋₃₃₉ peptide (o/p) or 10 μ g OVA₃₂₃₋₃₃₉ peptide/10 μ g OVA₃₂₃₋₃₃₉ peptide (p/p). Blood is collected at 3 hours after antigen injection on days 0 and 14. INF- γ levels are analyzed in the sera by an ELISA. Results are expressed as mean pg per ml serum \pm SEM.

TABLE 1

administration scheme	INF- γ level in pg/ml serum after	
	day 0	day 14
a/a	12.077 \pm 7.455	32.203 \pm 2.201
o/o	3.788 \pm 1.495	6.796 \pm 6.189
o/p	14.023 \pm 3.871	577.546 \pm 148.064
p/p	483.215 \pm 134.641	554.136 \pm 110.652

The i.p. administration of alum alone results in an about 4 fold increase in INF- γ levels within 3 hours after the injection. A second injection of alum 2 weeks later induces a similar rise in INF- γ levels in the sera. The primary sensitization with 10 μ g of OVA does not change the serum INF- γ levels above those seen with alum alone. However, when these mice receive 10 μ g of OVA₃₂₃₋₃₃₉ peptide 2 weeks later, about a 25 fold rise in the INF- γ serum levels can be measured compared to mice which receive alum alone. A primary sensitization with OVA followed 2 weeks later by a second OVA exposure fail to increase the INF- γ levels about those determined in the sera before the second sensitization. Mice injected for the first time with 10 μ g of OVA₃₂₃₋₃₃₉ peptide respond with a 50 fold increase in INF- γ levels in their sera 3 hours after the peptide injection. The second sensitization with the same dose of OVA₃₂₃₋₃₃₉ peptide induces a rise of the INF- γ levels of the same magnitude.

These results show that an increase in serum INF- γ levels can be induced in DO11.10 mice by sensitization with the OVA₃₂₃₋₃₃₉ peptide but the serum levels are under the detection limit when mice are challenged with the given amount of OVA-protein.

b) Serum cytokine levels upon stimulation with various doses of OVA₃₂₃₋₃₃₉ peptide**1) Changes in serum INF- γ levels**

Serum INF- γ levels after primary and secondary stimulation with various doses of the OVA₃₂₃₋₃₃₉ peptide are summarized in **TABLE 2**: Groups of 6 mice receive various doses (1, 3, 10, 30 and 90 μ g/mouse) of alum-precipitated peptide i.p. on day 0 and 14. Mice of the control group are injected with alum. Blood is collected before and at 2, 4, and 6 hours after the first peptide injection. INF- γ is determined in the sera by an ELISA. Results are expressed as mean pg per ml serum \pm SEM.

TABLE 2

administration scheme	INF- γ level in pg/ml serum on day 0 after different time points			
	after 0 hours	after 2 hours	after 4 hours	after 6 hours
alum	3.559 \pm 1.296	7.256 \pm 3.133	10.347 \pm 1.423	14.166 \pm 2.464
1 μ g OVA ₃₂₃₋₃₃₉	11.181 \pm 1.288	18.047 \pm 3.944	41.034 \pm 12.394	15.925 \pm 4.594
3 μ g OVA ₃₂₃₋₃₃₉	8.230 \pm 1.430	62.724 \pm 7.939	63.023 \pm 12.462	25.962 \pm 8.858
10 μ g OVA ₃₂₃₋₃₃₉	0.374 \pm 0.324	65.927 \pm 23.147	202.260 \pm 48.410	24.047 \pm 9.455
30 μ g OVA ₃₂₃₋₃₃₉	3.988 \pm 0.700	207.727 \pm 62.093	176.807 \pm 79.268	187.325 \pm 62.370
90 μ g OVA ₃₂₃₋₃₃₉	—	407.899 \pm 106.513	413.925 \pm 65.247	514.918 \pm 72.758

In the control group, receiving alum alone, INF- γ levels do not change significantly when blood is collected before and at 2, 4, and 6 hours after the injection. The primary stimulation with a dose of 1 μ g of OVA₃₂₃₋₃₃₉ peptide per mouse induces a small increase in serum INF- γ levels only at 4 hours. A dose of 3 μ g of OVA₃₂₃₋₃₃₉ peptide increases serum INF- γ levels about 11 fold at 2 and 4 hours compared to the control group receiving alum diluted with saline. INF- γ levels in the serum increase about 40 fold at 4 hours after injecting 10 μ g of OVA₃₂₃₋₃₃₉ peptide while a 30 μ g of OVA₃₂₃₋₃₃₉ peptide dose per mouse results in a 32-38 fold increase at 2, 4 and 6 hours after peptide injection. Increasing the dose to 90 μ g of peptide per mouse increases the INF- γ serum concentration 75 fold at 2 and 4 hours and 94 fold at 6 hours after injection. A similar picture of the rises of the serum INF- γ levels is seen when the animals are stimulated with the same doses of the OVA₃₂₃₋₃₃₉ peptide 2 weeks later.

2) Serum IL-4 levels

Serum IL-4 levels after primary and secondary OVA₃₂₃₋₃₃₉ peptide antigen stimulation are shown in **TABLE 3**: Groups of 6 mice receive various doses (1, 3, 10, 30 and 90 μ g/mouse) of alum-precipitated OVA₃₂₃₋₃₃₉ peptide i.p. on day 0 and 14. Animals of the control group are injected with alum. Blood is collected before and at 2, 4, and 6 hours after the first (A) and after the second peptide injection (B). IL-4 is determined in the sera by an ELISA. Results are expressed as mean pg per ml serum \pm SEM.

TABLE 3

administration scheme	IL-4 level in pg/ml serum on day 0 after			
	0 hours	2 hours	4 hours	6 hours
alum	14.865 +/- 1.384	31.994 +/- 5.465	19.173 +/- 1.902	17.891 +/- 1.914
1 µg OVA ₃₂₃₋₃₃₉	25.770 +/- 2.618	36.426 +/- 5.871	4.094 +/- 1.072	13.354 +/- 4.010
3 µg OVA ₃₂₃₋₃₃₉	17.905 +/- 3.616	37.706 +/- 4.028	7.903 +/- 0.957	11.051 +/- 2.549
10 µg OVA ₃₂₃₋₃₃₉	16.296 +/- 3.397	41.870 +/- 9.751	18.804 +/- 1.757	5.735 +/- 2.226
30 µg OVA ₃₂₃₋₃₃₉	16.524 +/- 2.209	93.594 +/- 29.015	41.725 +/- 10.226	17.173 +/- 2.069
90 µg OVA ₃₂₃₋₃₃₉	—	273.017 +/- 95.997	146.940 +/- 21.784	127.616 +/- 7.397

In mice of the control group, injected with alum, IL-4 levels remain unchanged as is also seen in the mice which receive 1 µg of peptide. Doses of 3 µg and 10 µg of peptide increase serum IL-4 levels only slightly after 2 hours compared to the control animals injected with alum. Serum IL-4 levels rise 3 fold at 2 hours after the administration of a dose of 30 µg of peptide while a 90 µg peptide dose induces a 14 fold increase after 2 hours and a 7-8 fold rise after 4 and 6 hours. The second injection of 1µg of peptide 2 weeks later does not change the IL-4 levels significantly when compared to the control.

3) Serum IL-2 levels

Serum IL-2 levels after primary OVA₃₂₃₋₃₃₉ peptide antigen stimulation are shown in TABLE 4: Groups of 6 mice receive various doses (3, 10, 30 and 90 µg/mouse) of alum-precipitated OVA₃₂₃₋₃₃₉ peptide i.p. on day 0. Animals of the control group are injected with alum. Blood is collected before and at 2 hours after the peptide injection. IL-2 is determined in the sera by an ELISA. Results are expressed as mean pg per ml serum ± SEM.

TABLE 4

administration of	IL-2 levels in pg/ml serum on day 0 after 2 hours
alum alone	135.939 +/- 17.348
3 µg OVA ₃₂₃₋₃₃₉	327.513 +/- 76.331
10 µg OVA ₃₂₃₋₃₃₉	2866.966 +/- 361.613
30 µg OVA ₃₂₃₋₃₃₉	2926.303 +/- 381.536
90 µg OVA ₃₂₃₋₃₃₉	4841.034 +/- 108.352

The results of this study demonstrate a dose/amount-dependent rise in serum IFN-γ, IL-4 and IL-2 levels with maximum values at several hours after the injection of the OVA₃₂₃₋₃₃₉

peptide. It seems that even with a dose of 90 µg of peptide, the plateau of the dose-response relationship of the serum cytokine levels is not reached. A dramatic rise in the IL-4 levels up to several 100 fold can be seen after the second antigen stimulation.

c) Sensitization with CsA before stimulation with OVA₃₂₃₋₃₃₉ peptide

- 5 The influence of CsA on cytokine production in DO11.10 mice after stimulation with the OVA₃₂₃₋₃₃₉ peptide is given in **TABLE 5**: Groups of 6 DO11.10 mice are pretreated orally with various doses (10, 20, 30, 40, 80 mg/kg) of CsA. One hour later 30 µg of alum precipitated OVA₃₂₃₋₃₃₉ peptide is injected i.p. Animals of the control group receive the solvent (placebo) of CsA. Blood is collected 2 hours later and IFN-γ, IL-4 and IL-2 levels are determined in the
10 sera by ELISAs. Results are expressed as mean pg per ml serum ± SEM.

TABLE 5

administration of	level of IFN-γ in pg/ml serum	level of IL-4 in pg/ml serum	level of IL-2 in pg/ml serum
placebo	468.721 +/- 79.315	342.129 +/- 71.157	3219.232 +/- 231.559
10 mg/kg CsA	423.781 +/- 118.481	111.515 +/- 37.08	2842.870 +/- 357.515
20 mg/kg CsA	56.675 +/- 19.957	3.982 +/- 3.093	758.934 +/- 149.960
30 mg/kg CsA	35.396 +/- 11.366	36.789 +/- 9.829	297.635 +/- 24.920
40 mg/kg CsA	8.958 +/- 5.351	5.339 +/- 2.552	149.981 +/- 30.115
80 mg/kg CsA	17.376 +/- 15.214	22.882 +/- 8.417	121.848 +/- 38.565

- As can be seen in **TABLE 5**, the IL-2 levels in the sera of mice receiving the CsA solvent
15 increases from pretreatment values below the detection limit to a mean value of 3219±231 pg/ml serum within 2 hours after the administration of 30 µg of OVA₃₂₃₋₃₃₉ peptide. Pretreatment with an oral dose of 10 mg of CsA/kg 1 hour before OVA₃₂₃₋₃₃₉ peptide reduces the IL-2 content by only 12% while 20 mg/kg reduces the IL-2 concentration by 76%. The IL-2 content of the sera when the mice are treated with 30 mg of CsA/kg b.w. is only 9% of the
20 control group. Doses of 40 and 80 mg/kg reduce the IL-2 content in the sera to about 5%. The reduction in the IFN-γ values in response to the CsA treatment is also shown. Within 2 hours after the administration IFN-γ serum level in the control mice rise from pretreatment values below the detection limit to a mean value of 468 ±79 pg/ml serum. The pretreatment with CsA at a dose of 10 mg/kg orally reduces the INF-γ serum levels to 90%. At a dose of
25 20 mg/kg b.w., IFN-γ levels drops to 12%. A further reduction to about 7% is seen at a dose of 30 mg/kg. Increasing the CsA dose to 40 and 80 mg/kg b.w. results in IFN-γ levels of 2% and 4% respectively. The IL-4 serum levels in response to various doses of CsA are shown.

IL-4 serum levels rise from non detectable values to a mean value of 342 ± 71 pg/ml serum in the control mice. A CsA dose of 10 mg/kg b.w. reduces the serum IL-4 concentration to 65%. The dose of 20 mg/ kg b.w. reduces the IL-4 levels in serum to 1.5% and at a dose of 30 mg/kg the IL-4 level is 10%. The CsA doses of 40 and 80 mg/ml has only 4% of the IL-4 measured in the control mice. The results of this experiment show that CsA administered orally at doses ranging from 10 to 80 mg/kg is very potent in reducing the antigen induced production of the cytokines IL-2, IFN- γ and IL-4 in DO11.10 mice. For IL-2 an ED₅₀ value of CsA of 14.3 ± 1.4 mg/kg is calculated. For the calculation of an ED₅₀ for IFN- γ and IL-4 additional doses have to be applied because of the steepness of the dose response curves.

Example 3: DO11.10 mice with priming and triggering agents

A) EXPERIMENTAL SETUP

a) Animals

Animals are provided as described in Example 2a).

b) Administration of OVA₃₂₃₋₃₃₉ (= OVA-peptide) and LPS

Groups of six DO11.10 mice receive i.p. 30 μ g of OVA₃₂₃₋₃₃₉ peptide (98% purity, pi Chem R&D, Graz Austria) in 0.2 ml 0.9% saline per mouse. Control mice are injected i.p. with the same amount of saline.

18 hours later the mice are challenged i.p. with 100, 300 or 500 μ g LPS (E.coli 0111: B4 Sigma Vienna, Austria). At 2, 4 and 6 hours after the LPS administration about 200 μ l of blood are collected by puncture of the retro-orbital plexus under ether anesthesia. The sera are collected and stored frozen at -20° until analyzed.

c) Quantification of cytokines

Murine IL-1 α , IL-1 β and TNF- α serum levels are determined by ELISAs (IL-1 α and TNF- α , Bender MedSystems, Vienna Austria; IL-1 β , Quantikine R&D Systems, Minneapolis, MN). Results are expressed as pg per ml serum. Differences in the serum cytokines concentrations between the different experimental groups are examined for statistical significance by the Mann-Whitney U test, two tailed. Results are expressed as the mean \pm SEM. $p < 0.05$ is regarded as significant.

B) RESULTS

1) IL-1 α serum levels

The injection of OVA-peptide at doses between 3 and 90 μ g per mouse increases serum concentrations of IFN- γ as well as other cytokines, such as IL-4 and IL-2, in a dose/amount-dependent way. Maximum concentrations, up to more then several 100 fold above

pretreatment values, are reached 2 to 4 hours after i.p. administration of 30 or 90 µg of OVA-peptide. Since no difference is found in the serum IFN-γ levels between doses of 30 and 90 µg of OVA-peptide, a dose of 30 µg is chosen for the priming of the DO11.10 mice. Values for serum IL-1α responses in control DO11.10 mice challenged with various doses of LPS and in mice primed with the OVA-peptide 18 hours prior to challenge are given in **TABLE 6**. The serum IL-1α levels are expressed as mean pg per ml serum ± SEM.

TABLE 6

administration of	IL-1α level in pg/ml serum after		
	2 hours	4 hours	6 hours
NaCl + 100µg LPS	38.699 +/- 5.074	45.230 +/- 8.495	24.874 +/- 7.586
NaCl + 300µg LPS	28.691 +/- 6.006	37.682 +/- 7.839	31.575 +/- 9.990
NaCl + 500µg LPS	28.691 +/- 6.006	37.682 +/- 7.839	31.575 +/- 8.880
OVA ₃₂₃₋₃₃₉ +100µg LPS	42.686 +/- 4.661	63.890 +/- 29.615	103.075 +/- 41.810
OVA ₃₂₃₋₃₃₉ +300µg LPS	36.833 +/- 9.638	171.013 +/- 54.849	192.811 +/- 61.966
OVA ₃₂₃₋₃₃₉ +500µg LPS	118.596 +/- 14.518	149.724 +/- 52.643	524.951 +/- 111.299

The treatment with LPS at doses of 100, 300 or 500 µg per mouse induces IL-1α serum levels in the range of 22.9±6.7 to 56.9±5.7 pg per ml serum. However, no dose dependency in the IL-1α serum levels can be detected in control mice when the sera are analyzed 2, 4 and 6 hours after the LPS challenge. OVA-peptide priming results in a rapid increase in IL-1α serum levels when challenged with the highest LPS dose.

2) IL-1β serum levels

Values of serum IL-1β responses in control DO11.10 mice challenged with various doses of LPS (=NaCl + LPS) and in mice primed with the OVA-peptide 18 hours prior to the LPS challenge (=OVA + LPS) are given in **TABLE 7**. The serum IL-1β levels are expressed as mean pg per ml serum ± SEM.

TABLE 7

administration of	IL-1β level in pg/ml serum after		
	2 hours	4 hours	6 hours
NaCl + 100µg LPS	252.303 +/- 68.712	295.269 +/- 35.911	153.009 +/- 73.919
NaCl + 300µg LPS	169.221 +/- 128.303	355.218 +/- 24.992	554.143 +/- 83.078
NaCl + 500µg LPS	148.578 +/- 8.674	558.418 +/- 30.431	680.661 +/- 57.453

OVA ₃₂₃₋₃₃₉ +100µg LPS	426.984 +/- 72.930	512.215 +/- 247.248	1198.531 +/- 392.919
OVA ₃₂₃₋₃₃₉ +300µg LPS	331.063 +/- 91.489	957.433 +/- 465.283	1712.753 +/- 655.367
OVA ₃₂₃₋₃₃₉ +500µg LPS	517.428 +/- 110.161	1223.519 +/- 475.491	3866.987 +/- 696.588

The IL-1 β serum levels in unprimed mice challenged with 100 µg of LPS are 252.3±68.7 pg/ml 2 hours after the challenge. The corresponding concentration in OVA-peptide primed mice is 1.6 fold higher. 4 hours after the LPS challenge, the concentrations are similar with a 1.7 fold increase. After 6 hours the values are 7.8 fold increased in OVA-peptide primed animals. The challenge of unprimed mice with 300 µg of LPS results in a mean concentration of 169.2±18.3 pg/ml and a 1.9 fold increase is found in the OVA-peptide primed mice after 2 hours. After 4 hours the IL-1 β concentration in the OVA-peptide primed mice rises 2.7 fold as compared to an unprimed mean. 6 hours after the LPS challenge, a 3.0 fold increase can be measured in the OVA-peptide primed animals. The LPS challenge with a dose of 500 µg per mouse induces a 3.5 fold rise in the OVA-peptide primed mice after 2 hours. After 4 hours IL-1 β levels are 2.2 fold higher. A substantial rise of a 5.7 fold increase in the IL-1 β levels can be detected in OVA-peptide primed mice 6 hours after the LPS challenge.

3) TNF- α serum levels

The effects of various doses of LPS (100, 300, or 500 µg/mouse) on the production of TNF- α in the serum of non-sensitized DO11.10 mice or mice sensitized by i.p. injection of OVA-peptide (30µg/mouse, 18 hours prior to LPS challenge) are given in **TABLE 8**. The TNF- α serum levels are expressed as mean pg per ml serum \pm SEM.

TABLE 8

administration of	TNF- α level in pg/ml serum after		
	2 hours	4 hours	6 hours
NaCl + 100µg LPS	672.286 +/- 619.156	95.660 +/- 24.845	188.909 +/- 169.018
NaCl + 300µg LPS	1144.708 +/- 286.542	567.901 +/- 432.819	39.641 +/- 11.835
NaCl + 500µg LPS	1505.555 +/- 204.078	162.556 +/- 34.477	241.637 +/- 142.935
OVA ₃₂₃₋₃₃₉ +100µg LPS	6552.911 +/- 2645.343	133.059 +/- 53.186	105.297 +/- 37.387
OVA ₃₂₃₋₃₃₉ +300µg LPS	8979.088 +/- 2907.689	385.924 +/- 194.462	253.396 +/- 136.190
OVA ₃₂₃₋₃₃₉ +500µg LPS	7891.200 +/- 2394.159	222.733 +/- 104.548	321.129 +/- 57.176

2 hours after the challenge with 100 µg of LPS, the TNF- α levels are 672±61.9 pg/ml in unprimed animals and 9.7 fold higher in OVA-primed mice. At a challenge dose of 300 µg of

LPS, a mean of 1144.708 ± 286.5 pg/ml can be determined in the sera of unprimed animals. The OVA-peptide priming increases the TNF- α concentration 7,8 fold. OVA-peptide priming before challenged with 500 μ g of LPS increases the amount 5.2 fold. 4 hours after the challenge with 100 μ g of LPS, the TNF- α levels decline to 95.6 ± 24.8 pg/ml in unprimed mice.

5 In OVA-primed animals no significant changes in the TNF- α levels can be determined (133.0 ± 53.1 pg/ml). A similar effect of no difference in the TNF- α levels between unprimed and OVA-peptide primed animals is seen at LPS challenge doses of 300 μ g.

6 hours after the LPS challenge, the TNF- α concentrations in the sera of the OVA-primed mice are 105.2 ± 37.3 pg/ml at 100 μ g of LPS; 253.4 ± 136.2 pg/ml at 300 μ g of LPS and

10 321.1 ± 57.1 pg/ml at 500 μ g of LPS.